Aging and Disease
www.aginganddisease.org
Volume 3, Number 2: 164-170; April 2012

Origianl article

The Subtelomere of Short Telomeres is Hypermethylated in Alzheimer's Disease

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[Received October 7, 2011; Revised November 30, 2011; Accepted November 30, 2011]

ABSTRACT: Telomere shortening has been reported to be related to oxidative stress (OS) associated with the aging process and aging-associated diseases, including Alzheimer's disease (AD). We measured the methylated and non-methylated telomere lengths in the peripheral blood mononuclear cells of 34 AD patients and 49 healthy controls by a Southern blotting analysis, using methylation-sensitive and -insensitive restriction enzyme isoschizomers, *Msp*I and *Hpa*II. AD patients bore normal mean telomere lengths and had an unchanged distribution of the telomere length in peripheral leukocytes. However, the subtelomeres in the shortest telomeres were relatively more methylated in AD patients of both genders, compared with age-matched controls. We observed that the pathogenesis of AD was associated with the epigenetic condition of the subtelomere, but not on the overall telomere length and distribution. The relative elevation of subtelomeric methylation of short telomeres in peripheral blood leukocytes may be a characteristic of AD. This implies that leukocytes containing short telomeres with less methylated subtelomeres tend to be removed faster from the peripheral blood in AD patients.

Key words: Alzheimer's disease; Telomere; Subtelomere; DNA methylation

The terminus of genomic DNA in eukaryotic cells contains thousands of conservative repeats of pentamer or hexamer units [1,2]. This forms a protective structure with various accessory protein factors, which is called a telomere. The telomere DNA sequence consists of repeats of TTAGGG/AATCCC in humans. The telomere structure functions to protect the genomic DNA from exonuclease attacks or end-to-end DNA recombination between different chromosomes which could lead to carcinogenesis. Telomere shortening is an aging-related genomic change in somatic cells. The mean telomere lengths (TRF) of peripheral leukocytes are known to become shorter with aging [3-6]. As a result of unidirectional DNA synthesis during DNA duplication at mitosis, the daughter DNA is slightly shorter due to a small loss of the terminal DNA sequence. Telomeres become shorter little by little during each cell cycle. In addition, telomere shortening is accelerated by various disease conditions, such as mental stress, obesity, smoking, type 2 diabetes mellitus, ischemic heart diseases, Alzheimer's disease (AD) and Parkinson's disease [7-11]. In these pathological conditions, abnormally enhanced local or systemic oxidative stress contributes to telomere-erosion, thus resulting in accelerated telomere shortening of local or systemic somatic cells.

The mean TRF of peripheral leukocytes of healthy people is shortened with aging, and the number of long

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ISSN: 2152-5250

telomeres decreases and that of short telomeres increases during aging-related telomere shortening [5]. AD is a major neurodegenerative disorder. As the elderly population increases, the prevalence of age-related diseases, such as AD, also increases. The incidence and prevalence of AD increase dramatically in subjects over age 60, and the disease has an approximately 50% prevalence for patients over age 85 [12]. A large body of evidence has confirmed the presence of localized oxygen stress (OS) in AD [13-15]; however, the etiology of AD is still poorly understood.

These observations led us to hypothesize that aging-associated telomere shortening is associated with an altered subtelomeric methylation status in humans, and that this is also affected by disease conditions such as AD. In the present study, the telomere length distribution and subtelomeric methylation status were analyzed in AD patients and compared to those in age-matched controls. Genomic DNA methylation is an indicator of the epigenetic status associated with the chromatin structure, i.e., heterochromatin or euchromatin. Recently, a relationship between subtelomeric DNA methylation and telomere shortening has been reported in both healthy subjects and Parkinson's disease patients [11, 16]. These observations strengthened our hypothesis that aging-associated telomere shortening is related to alterations of the subtelomeric methylation status, and that this phenomenon also affects AD patients.

MATERIALS AND METHODS

Study population

DNA samples from peripheral blood mononuclear cells (PBMC) (taken using 10 ml Vacutainer tubes containing EDTA/ heparinized syringes) of 49 Chinese healthy controls (65-76y.o.) and 34 Chinese AD patients (59-76y.o.) (Table 1), thus meeting the criteria of Diagnostic and Statistical Manual of Mental Disorders-IV diagnosis [17] whose score was between 10 and 26 on the Folstein Mini-Mental Status Exam (MMSE). PBMC samples were stored at -80°C until use. All patients were examined by a psychiatrist and a neuropsychologist during the admission procedure and also underwent routine laboratory tests, standard neuropsychologic examinations and cerebral perfusion study by single photon emission computed tomography. None of the AD subjects demonstrated any complications or other neurologic or mental diseases, and no evidence of any developmental abnormalities or significant neurologic antecedents. The subjects were excluded from the study if they had an inflammatory disease, were affected by diabetes mellitus, were on estrogen replacement therapy, or had been treated with vitamins. All subjects were nonsmokers. AD and controls were matched for age, dietary habits. Informed written consent was obtained from each subject. The present study was approved by the local Human Ethics Committee, and written consent was obtained from all the participants.

Telomeric Length Measurement

Telomere detection was performed as previously described with a modification [5,11,18]. Methylation-sensitive and methylation-insensitive isoschizomer *Hpa*II and *Msp*I were used as our previous studies [19-21]. Both enzymes recognize and cut tetranucleotide CCGG, but *Hpa*II does not cut CCGG with methylated cytosine of the dinucleotide CG in the center of CCGG. Briefly, genomic DNA was extracted from peripheral leukocyte specimens using PureGene DNA Extraction Kits (Gentra Systems, Minneapolis, MN), and the quality was assessed by agarose gel electrophoresis. The DNA (0.1μg) was digested at 37ºC with 1U *Msp*I or *Hpa*II for 2 h. The digests (10μl) were resolved by agarose gel-electrophoresis, and transferred by Southern blotting to a positively charged nylon membrane (Roche Diagnostics, Mannheim, Germany). The blotted DNA fragments were hybridized to a long (TTAGGG)n digoxigenin-labeled probe specific for telomeric repeats. The telomeric repeat probe used here is 500bp long. This length is much longer than that of conventional oligonucleotide telomere probes commonly used. This long probe yields dense signals and enables one to clearly detect telomeres shorter than 4.4kb according to a Southern blot analysis. The blotted membranes were incubated with anti-digoxigenin-alkaline phosphatase-specific antibody. The telomere probe was visualized by CSPD ($C_{18}H_{20}ClO_7PNa_2$) (Boehringer Mannheim GmbH, Mannheim, Germany). The membrane was then exposed to Fuji XR film with an intensifying screen (FUJIFILM Corporation, Tokyo, Japan). The smears of the autoradiogram were captured on an Image Master (Trioptics Japan, Shizuoka), and then the telomere length was quantitatively assessed.

If the difference within the two experiments was greater than 5%, we planned to measure it again and use the mean value of the three experiments. However, the difference within each set of duplicate experiments was always within 5%.

Terminal length analysis

The mean TRF (Terminal Restriction Fragment Length) was estimated using the formula Σ(ODi-background) /Σ(ODi-background/Li) [5], where ODi is the chemiluminescent signal and Li is the length of the TRF fragment at position i. A loss of a few hundred base pairs

from short telomeres could be important to cellular ageing but may not be detected by traditional mean TRF analysis [22, 23]. We compared telomere length using telomere percentage analysis with three intervals of length as defined by a molecular weight standard. In brief, the intensity (photo-stimulated luminescence: PSL) was quantified as follows: each telomeric sample was divided into grid squares as follows according to the molecular size ranges: >9.4, 9.4-4.4 and <4.4kb. The percent of PSL in each molecular weight range was measured (%PSL=intensity of a defined region-background × 100/total lane intensity-background). Telomeric methylation was assessed by the comparison between the *Msp*I telomere length distribution and that of *Hpa*II. The difference between the percent of *Msp*I PSL (%*Msp*I-TRF) and the *Hpa*II PSL (%*Hpa*II-TRF) in each molecular weight range was calculated. The proportion of the calculated difference (%*Hpa*II-TRF-%*Msp*I-TRF) in >9.4kb range to %*Hpa*II-TRF in >9.4kb range ((*Hpa*II-*Msp*I)/*Hpa*II(>9.4kb)) was used to evaluate the methylation status of telomeres longer than 9.4kb. Similarly, the proportion of the calculated difference (%*Msp*I-TRF-%*Hpa*II-TRF) in <4.4kb range to %*Msp*I-TRF in <4.4kb range ((*Msp*I-*Hpa*II) /*Msp*I(4.4kb>)) was used to evaluate the methylation status of telomeres shorter than 4.4kb.

Statistical Analysis

The normality of the data was examined with the Kolmogorov–Smirnov test and the homogeneity of variance with the Levene Median test. If both the normal distribution and equal variance tests were passed, the differences in the telomeres length including the mean TRF length and the telomere percentage analysis with age and condition (AD patients or age-matched healthy controls) were studied using a two-way analysis of variance (ANOVA) test followed by all pairwise multiple comparison procedures using Tukey's post hoc test. The data are expressed as the mean ± standard deviation. The criterion for the significance is $p<0.05$. All analyses were carried out using a Sigma Statistical Analysis software package (Sigma 2.03, 2001; St. Louis, MO).

Table 1. The age, mean TRF and the subtracted TRF of controls and AD patients

	Controls		AD patients		p-value			
	Male	Female	Male	Female	Control (M/F)	AD (M/F)	Male (C/AD)	Female (C/AD)
Number	26	23	17	17	0.763	1.0	0.332	0.507
Age (y.o)	68.7±2.8	72.1±4.9	67.4±5.6	71.6±4.0	0.604	0.126	0.359	0.109
***Msp*I-TRF (kb)**	6.0±0.8	6.3±0.9	6.2±0.5	6.1±0.9	0.442	0.564	0.391	0.509
***Hpa*II-TRF (kb)**	6.8±0.8	6.9±1.1	7.4±1.0	7.0±1.2	0.820	0.286	0.026	0.316
***H-M*-TRF (kb)**	0.7±0.4	0.6±0.5	1.2±0.7	0.9±0.6	0.091	0.231	0.010	0.616

H-M-TRF: *Hpa*II-*Msp*I-subtracted TRF, M/F: Gender-associated difference, C/AD: AD-associated difference.

RESULTS

The age and the mean *Msp*I-TRF of AD patients were similar to those of the controls. The mean *Hpa*II-TRF and the mean differences between the *Hpa*II-TRF and *Msp*I-TRF were larger in male AD patients, compared to controls (Table 1). This observation suggested that the subtelomeric region was more methylated in male AD patients than in either female AD patients or in control subjects. In our analysis of the telomere length distribution in AD patients, no significant differences were observed in the *Msp*I-TRF distribution between AD patients and controls (Figure 1A). However, in the *Hpa*II-TRF distribution in the males, the AD patients bore fewer short telomeres (<4.4kb), compared with the controls (Figure 1B). These results seemed to imply that short telomeres were apparently less methylated in male AD patients.

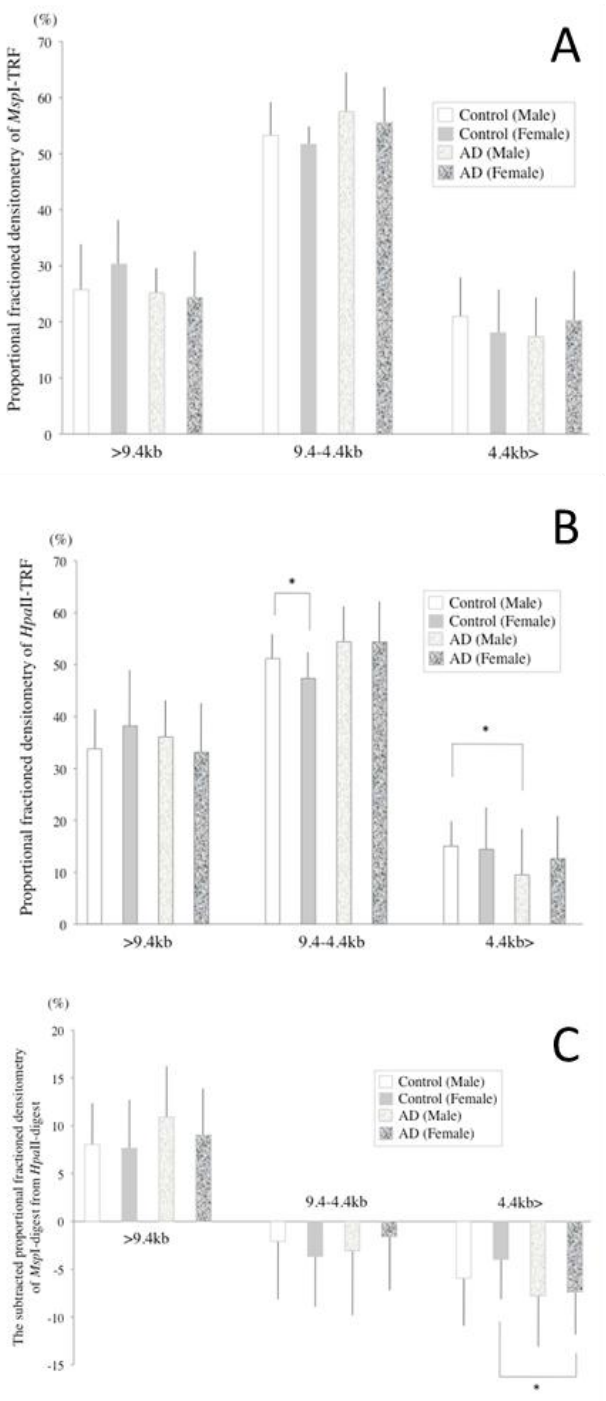

Figure 1. The telomere length distributions of *Hpa*II-TRF and *Msp*I-TRF, and the subtracted distribution in AD and control subjects. A, B. The changes in the subdivided *Msp*I-TRF (A) and *Hpa*II-TRF (B) distributions. The Southern blotting smears of *Hpa*II-TRF and *Msp*I-TRF were divided into three portions (>9.4kb, <9.4 but >4.4kb, <4.4kb). The percentages of the densitometry of each portion are shown as columns. Vertical bars depict the standard deviations. C. The subtracted *Hpa*II-*Msp*I TRF length distribution. The subtracted values of the *Msp*I-TRF from the *Hpa*II-TRF densitometry in the three subdivided parts are shown as columns. Vertical bars depict the standard deviations. *p<0.05 vs control.

In order to detect methylation-associated changes, the subtracted distribution was analyzed. In the subtraction of the *Msp*I-TRF distribution from the *Hpa*II-TRF distribution, the area difference in the short range (<4.4kb) seemed to significantly increase in the female AD patients (Figure 1C). In this analysis, the subtelomeres of short telomeress were more methylated in the female AD patients. Therefore, these analyses yielded confusing results with regard to the male and female patients and their methylation status.

However, it is known that the subtracted *Hpa*II-*Msp*I value can be affected by two other factors; namely, differences in the telomere length and in the methylation status. Therefore, in order to analyze the methylation status more specifically, the ratio of the subtraction of the *Msp*I-TRF lengths from *Hpa*II-TRF lengths of >9.4kb and <4.4kb to that of *Hpa*II-TRF lengths >9.4kb ((*Hpa*II-*Msp*I)/*Hpa*II(>9.4kb)) and to *Msp*I-TRF lengths <4.4kb ((*Msp*I-*Hpa*II)/*Msp*I(<4.4kb)) were calculated as methylation-specific parameters (Figure 2). The methylation level in the longest telomere length range and that in the shortest range are reflected by ((*Hpa*II-*Msp*I)/*Hpa*II(>9.4kb)) and ((*Msp*I-*Hpa*II)/*Msp*(<4.4kb)), respectively. The ratio of ((*Hpa*II-*Msp*I)/*Hpa*II (>9.4kb)) did not differ substantially between AD patients and the control subjects. On the other hand, the ratio of (*Msp*I-*Hpa*II)/*Msp*I (<4.4kb) was significantly higher in AD patients than in the controls in both genders. These results indicated that the subtelomeres of short telomeres are hypermethylated in AD patients of both genders compared to the control subjects.

DISCUSSION

AD is an aging-associated neurodegenerative disease, which is characterized by a progressive cognitive decline and memory impairment [24]. Recent reports have shown oxidative stress (OS) to be involved in the pathogenesis of AD [13-15]. The increased oxidative stress in AD patients contributes to telomere erosion, thus yielding accelerated telomere shortening of local or systemic somatic cells [8]. The telomere length has been reported to be abnormally shortened in AD patients [8]. However, the level of cognitive impairment and dementia in patients with Alzheimer's disease or vascular dementia is not always associated with the telomere length of leukocytes [25,26]. Tissue variations in the telomere length have also been reported, as telomeres are shortened in leukocytes and buccal cells, whereas they are elongated in the brain tissue of AD patients [27].

In our present analysis, the mean TRF and the *Msp*I-TRF distribution of AD patients did not differ

significantly from those of the controls. A decrease in the mean non-methylated TRF, a decrease in the long telomeres, and an increase in the short telomeres have been reported as aging-associated telomere changes [5,19]. However, none of these telomere length-associated changes were detected in the AD patients in this study. This result apparently indicated that the pathophysiological condition of AD did not affect the aging process by leading to the telomere shortening of somatic cells. However, the subtelomeric methylation status was affected in AD patients.

In the present study, the subtracted TRF and the subtracted TRF distribution of *Msp*I and *Hpa*II showed different results. The former showed AD-associated hypermethylation of the subtelomeres only in males, while the latter showed an increase in the subtelomeric methylation level of the shortest telomere range only in females. The proportion of subtelomere methylation was subsequently analyzed separately for the longest and the shortest telomere ranges, which lessened the bias caused by the interpersonal telomere length variations. The proportional rate of methylated subtelomeres in the shortest telomere range was increased in AD patients, irrespective of gender. Therefore, the proportional comparison of *Msp*I- and *Hpa*II-telomere lengths was more specific and sensitive for altered subtelomeric methylation than the simple comparison of the absolute values of these findings.

There are two possible explanations for the increased methylation level of the subtelomeres in the shortest telomere range. One is that AD-associated pathophysiological conditions enhance the methylation in subtelomeres as the telomere length shortens. The other is that the shorter telomeres containing less methylated subtelomeres tend to be lost faster in AD patients. A recent report showed that telomere attrition leads to subtelomeric hypomethylation. For example, a mouse mutant deficient in telomere-elongating activity, *terT*$^{-}$*/terT*$^{-}$, had detectable telomere shortening in descendent generations, with increased subtelomeric hypomethylation [30]. Therefore, the provocation of subtelomeric methylation of shortened telomeres is unlikely.

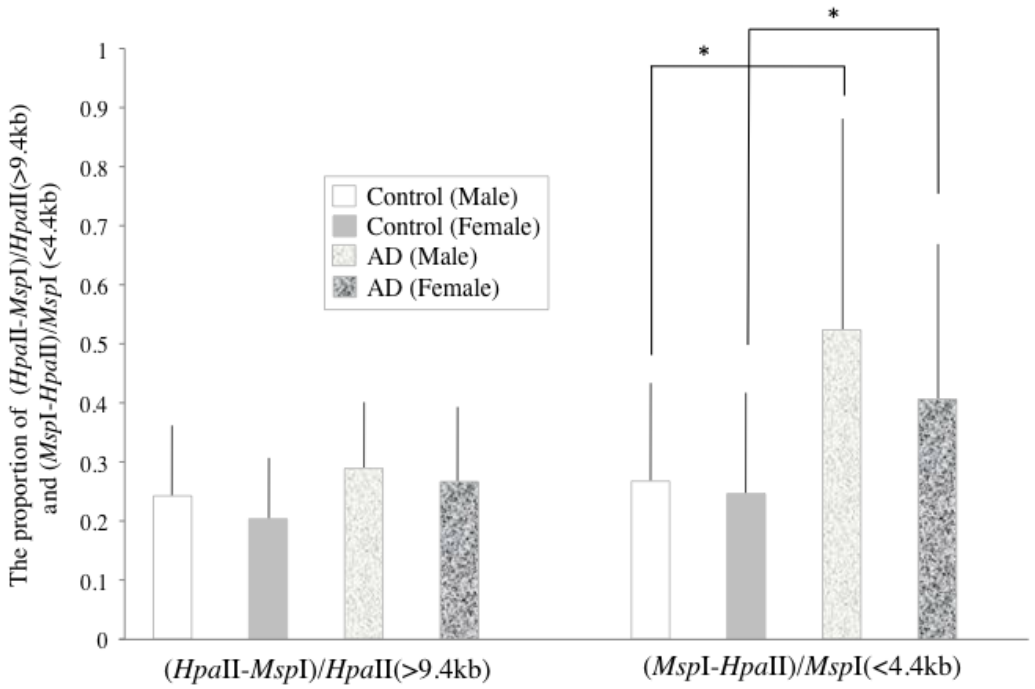

Figure 2. The changes in the relative methylation status of subtelomeres in AD patients. (*Hpa*II-*Msp*I)/*Hpa*II(>9.4b) and (*Msp*I-*Hpa*II)/*Msp*I(<4.4kb) were used as indices indicating the subtelomeric methylation of longer (than 9.4kb) and shorter (than 4.4kb) telomeres, respectively. Vertical bars depict the standard deviations. *p<0.05 vs control.

Increased OS has been suggested to be associated with the pathogenesis of AD [28,29]. The increased OS in AD patients would contribute to somatic telomere length attrition, thus leading to a decrease in the number of the longest telomeres and an increase in the number of the shortest telomeres, similar to the changes that normally occur with aging. If this is the case, then the longest telomeres would decrease, while the number of the shortest telomeres would increase in AD patients compared to the controls. No AD-associated accelerated telomere attrition was detected in either gender in this study. However, if cells bearing shorter telomeres with hypomethylated subtelomeres tended to be lost, then the expected increase in the shorter telomeres in AD patients would not be apparent.

OS generates hydroxyl radicals, which may trigger DNA damage, such as base modifications, deletions, strand breakage and chromosomal rearrangements, or may yield oxidized DNA products such as 8-hydroxy-deoxyguanosine and O^6-methylguanine [31]. The access of the DNA methyltransferase to subtelomeres may also be affected by DNA oxidation, thus resulting in subtelomeric hypomethylation [19]. These results were in apparent opposition to the observed telomere changes of the AD patients in this study, which showed an increase in the hypermethylation of the shortest telomeres. These results implied that it was more likely that short telomeres with hypomethylated subtelomeres tended to be lost, rather than that subtelomeric methylation of the shortest telomeres was enhanced in AD patients, where OS is generally thought to be elevated.

A tendency to lose the shortest telomeres has previously been reported in patients with Parkinson's disease and other pathological conditions [11,16,22,23]. A similar mechanism may therefore also be responsible, at least in part, for the telomeric changes observed in AD patients. Therefore, the alterations in the subtelomeric methylation status may be useful as a sensitive marker of the disease-associated effects of neurodegenerative disorders such as AD on the genomic DNA, even though the mean telomere length or the telomere length distribution of somatic cells appears to be unaltered in these patients.

To confirm the findings of our study, further analyses will be necessary to clarify whether cells showing increased short telomeres with subtelomeric hypomethylation under hyperoxidative conditions are prone to undergo cell death in patients with other chronic disease conditions, or under culture conditions.

Acknowledgments

We are grateful to Mr. Brian Quinn for his linguistic advice. This work was supported by the National Natural Science Fund (NSFC) (81170329/H2501) and by a Grant-in-Aid from the Ministry of Education, Science, and Culture of Japan (#23590885).

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